Charles University in Prague

Faculty of Pharmacy in Hradec Kralove

Department of Pharmacology and Toxicology

Neuroprotective and antioxidant effects of a series of coumarins in *in vitro* Alzheimer's disease models

Rigorous Thesis

Supervisor: PharmDr. Martina Čečková, Ph.D.

Supervisor U. Bologna: Prof.Asoc. Andrea Tarozzi

PhD. Student Letizia Pruccoli

2016 **Mgr. Lucie Pospěchová**

Statement of originality

I declare that this thesis is my own, original, personal work. All literature and other resources I used while processing are listed in the reference list and are properly cited.

Date: 14.11.2016 Mgr. Lucie Pospěchová

Acknowledgments

I would like to thank to my supervisor Doc. PharmDr. Andrea Tarozzi, CSc. for his advice and to Ph.D. Student Letizia Pruccoli for her guidance during this research and all her advices. My great thanks belong to PharmDr. Martina Čečková, Ph.D. for her advices and consultations about this work. I also want to thank her for allowing me to write this thesis at the Department of Pharmacology and toxicology. My special thanks go to my partner, family and friends for their support during my studies at the faculty and enhancing my motivation.

Content

1 Abstrakt

Univerzita Karlova v Praze

Farmaceutická fakulta v Hradci Králové

Katedra farmakologie a toxikologie

Studentka :	Mgr. Lucie Pospěchová
Školitel :	Doc. PharmDr. Andrea Tarozzi, CSc. PharmDr. Martina Čečková, Ph.D.
Název rigorózní práce :	Neuroprotektivní a antioxidační efekty látek ze skupiny kumarinů na *in vitro* modelech Alzherimerovy nemoci

Oxidativní stres je jedním z významných faktorů, které vedou k poškození a smrti neuronálních buněk. Děje se tak v důsledku vzniku reaktivních kyslíkatých radikálů, které se podílejí na patogenezi neurodegenerativních onemocnění, např. Alzheimerovy choroby (AD).

Vědecké studie dokazují, že přírodní kumariny vykazují antioxidační vlastnosti, a mohly by tudíž představovat nové možnosti léčby neurodegenerativních chorob. Esculetin (ESC), scopoletin (SCOP), fraxetin (FRAX) a daphnetin (DAPH) patří do skupiny fenolických kumarinů a vzhledem ke katecholové skupině a α-pyronovém kruhu vykazují značné antioxidační vlastnosti.

V této práci jsme se zabývali antioxidačními a neuroprotektivními vlastnostmi ESC, SCOP, FRAX a DAPH na lidských neuronálních buňkách (SH-SY5Y). Zkoumali jsme aktivitu proti 2,2-difenyl-1-pikrylhydrazylu (DPPH), vzniku reaktivních kyslíkatých radikálů a cytotoxicitě způsobené *terc*-butyl hydroperoxidem (t-BuOOH). Dále byla testována schopnost ESC zvýšit hladiny cytosolového glutationu (GSH) a inhibovat cytotoxicitu způsobenou oligomery $A\beta_{1-42}$ ($OA\beta_{1-42}$).

2 Abstract

Charles University in Prague

Faculty of Pharmacy in Hradec Kralove

Department of Pharmacology and Toxicology

Student:	Mgr. Lucie Pospěchová
Supervisor:	Doc. PharmDr. Andrea Tarozzi, CSc. PharmDr. Martina Čečková, Ph.D.
Title of rigorous thesis:	Neuroprotective and antioxidant effects of a series of coumarins in *in vitro* Alzheimer's disease models

Oxidative stress represents one of the crucial factors leading to the neural cell damage and death. This process occurs due to generation of reactive oxygen radicals that contribute to the pathogenesis of neurodegenerative disorders such as Alzheimer's disease (AD). Recent scientific studies have reported that natural coumarins possess antioxidant properties and might therefore represent new treatment option in the therapy of neurodegenerative diseases including AD. In particular, phenolic coumarins comprising esculetin (ESC), scopoletin (SCOP), fraxetin (FRAX) and daphnetin (DAPH), possess considerable antioxidant activities due to the catechol group and the α-pyrone ring.

In this study, we investigated the antioxidant and neuroprotective effects of ESC, SCOP, FRAX and DAPH using human neuronal (SH-SY5Y) cells and evaluated activity of these compounds against DPPH and ROS formation and cytotoxicity induced by tert-butyl hydroperoxide (t-BuOOH). In addition, we tested the ability of ESC to increase cytosolic glutathione (GSH) levels and inhibit the cytotoxicity induced by $A\beta_{1-42}$ oligomers ($OA\beta_{1-42}$).

3 List of Abbreviations

Ach	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer disease
APP	Amyloid precursor protein
Aβ	β-amyloid protein
BACE1	β-secretase enzyme
BDNF	Brain-derived neutrophic factor
CNS	Central nervous system
CYP 2A6	Cytochrome P-450-linked mono-oxygenase
DAPH	Daphnetin
D'PBS	Dulbecco's phosphate buffered saline
DMSO	Sterile dimethylsulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
ESC	Esculetin
EtOH	Ethanol
FBS	Fetal bovine serum
FRAX	Fraxetin
GH	Growth hormone
GMCSF	Granulocyte macrophage colony-stimulating factor
GSH	Glutathione
GSSG	Glutathione disulfide
HBSS	Hank's balanced salt solution
IDE	Insulin-degrading enzyme
IL-1α	Interleukin 1α
IL-6	Interleukin 6
Keap1	Kelch-like ECH-associated protein 1
MAO	Monoamine oxidase
MTT	Salts of tetrazole
Nrf2	NF-E2 related factor 2
$OA\beta_{1-42}$	Oligomers of $OA\beta_{1-42}$
ROS	Reactive oxygen species
SCOP	Scopoletin
SH-SY5Y	Cells of neuroblastoma
t-BuOOH	Tert-butylhydroperoxide
TNFR1	TNF Receptor 1
TNFR2	TNF Receptor 2
TNFα	Tumor necrosis factor α

4 Introduction

Alzheimer's disease (AD) is one of the most common neurodegenerative illnesses. Its incidence increases with age (with the highest frequency around the age of 65). It is characterized by a memory loss, poor sleep quality, emotional changes, anxiety, depression and cognitive deterioration.[1]

AD is a multifactorial disease with several mechanisms including processing of amyloid precursor protein (APP) to amyloid β peptide, hyperphosphorylation of tau protein, abnormal spiral filaments in neurons and increased oxidative stress. Neurotransmitters such as glutamate and serotonin are also affected during AD.[1,2]

There is also an interest in mechanisms propagated by obesity, type-2 diabetes mellitus, metabolic syndrome, stress, sedentariness and dietary overconsumption of saturated fat and refined sugars.[1]

AD correlates with the disproportional loss of synapses between neurons, while neurofibrillary tangles of hyperphosphorylated protein tau are directly related to the severity of AD and they can serve as a pathological marker.[1]

The deficiency in the neurotransmitter acetylcholine (ACh) and cholinergic neurotransmission dysfunction in brain of AD patients seems to play a fundamental role in a memory deterioration. Acetylcholinesterase is the enzyme that degrades ACh, and accelerates amyloid-β peptide formation, which is one of the pathological sign of the disease.[3]

Unfortunately, there is no effective cure for AD available so far and current treatment strategies resolve primarily symptomatic problems associated with AD. Efforts to develop new therapeutic approaches are focused on inhibition of acetylcholinesterase (AChE) to improve cholinergic neurotransmission between neurons.[2]

Several of the cholinesterase inhibitors, that are currently in use, like donepezil, rivastigmine, galantamine and tacrine, have some adverse side-effects; e.g. gastrointestinal disorders, nausea and vomiting, therefore there has been a scientific effort in researching natural inhibitors of AChE, butyrylcholinesterase (BChE) and beta-site APP cleaving enzyme 1 (BACE1) with safer profiles.[2,4]

Natural compounds with "multi target" mechanisms could be important in the development of new compounds with neuroprotective features.

Recent studies demonstrated that exactly coumarins possess this neuroprotective ability. Coumarins are also able to inhibit enzymes such as MAO, AChE and γ-secretase. They also show the effect against excitotoxicity mediated by glutamate. Recently they showed also their capacity to promote the release of neuroprotective factors like brain-derived neutrophic factor (BDNF) in the cerebral cortex and to oppose neuroinflammatory processes by reducing the release of pro-inflammatory cytokines and neurotoxic factors that are induced by Aβ protein.

5 Theoretical part

5.1 Mechanisms involved in Alzheimer's disease

5.1.1 β-amyloid protein

β-amyloid protein (Aβ) is derived from a transmembrane amyloid precursor protein (APP). APP is metabolized by enzymatic actions of BACE-1, α-secretase and γ-secretase. Aβ peptides are formed as a result of these enzymatic reactions and they are constituted from 36 to 43 amino acids. Specific peptides are formed due to the action of γ-secretase. $A\beta_{40}$ is the most common form of this peptide. On the other hand, $A\beta_{42}$ is more prone to aggregation and consequent **extracellular** formation of multimers or fibrils.[5] These amyloid deposits are surrounded and attacked by reactive astrocytes and microglia. This process can induce irreversible neuronal cell death and cognitive impairment, loss of memory and physical deterioration. These senile plaques are formed principally in hippocampus, amygdala and cortex.[1]

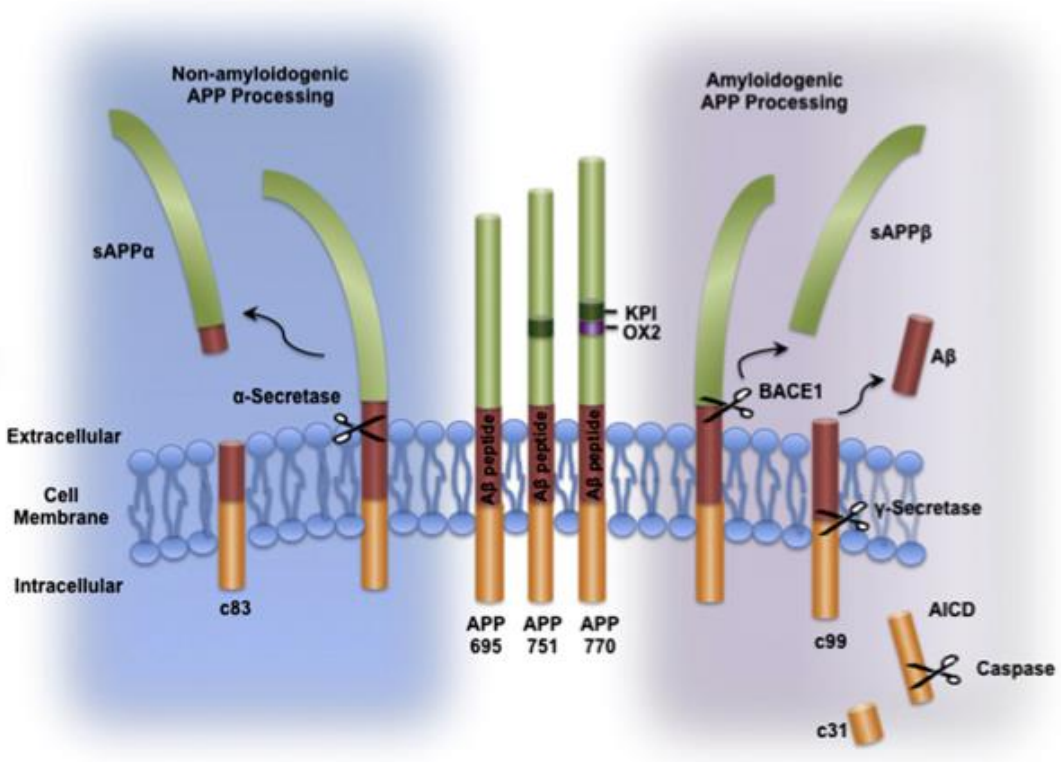


Fig. 1: Cleavage of APP into β-Amyloid by BACE-1, α-secretase and γ-secretase.[6]

A possible approach to reduce β-amyloid formation is to inhibit β-secretase (BACE1) which is a transmembrane enzyme responsible for the production of β-amyloid peptide. Inhibition of AChE and BChE (the enzyme similar to AchE, which also plays an important role in the pathogenesis of AD) is also an effective drug strategy to treat AD.[3]

Aβ is degraded by the insulin-degrading enzyme (IDE), the same one that decomposes insulin. High levels of insulin then influence Aβ clearance.[1]

In addition, growth hormone (GH) released from the anterior pituitary can influence repairing processes and growing in CNS. The activity of this hormone declines with age. A lower amount of GH correlates also with an increase of total body and visceral fat and decreased physical activity. There is also evidence that GH influences the CNS cognitive processes.[1]

5.1.2 Neurofibrillary Tangles

Formation of **intracellular** neurofibrillary tangles is another process that influences negatively the pathology of AD.[5]

The tangles are the result of an incorrect configuration of microtubules that stabilize protein tau. Neurofibrillary tangles are formed by paired helical filaments. They can accumulate within neuronal cells leading to a destabilization and dissociation of microtubules. This process can be caused by a synaptic failure particularly in the hippocampus.[1]

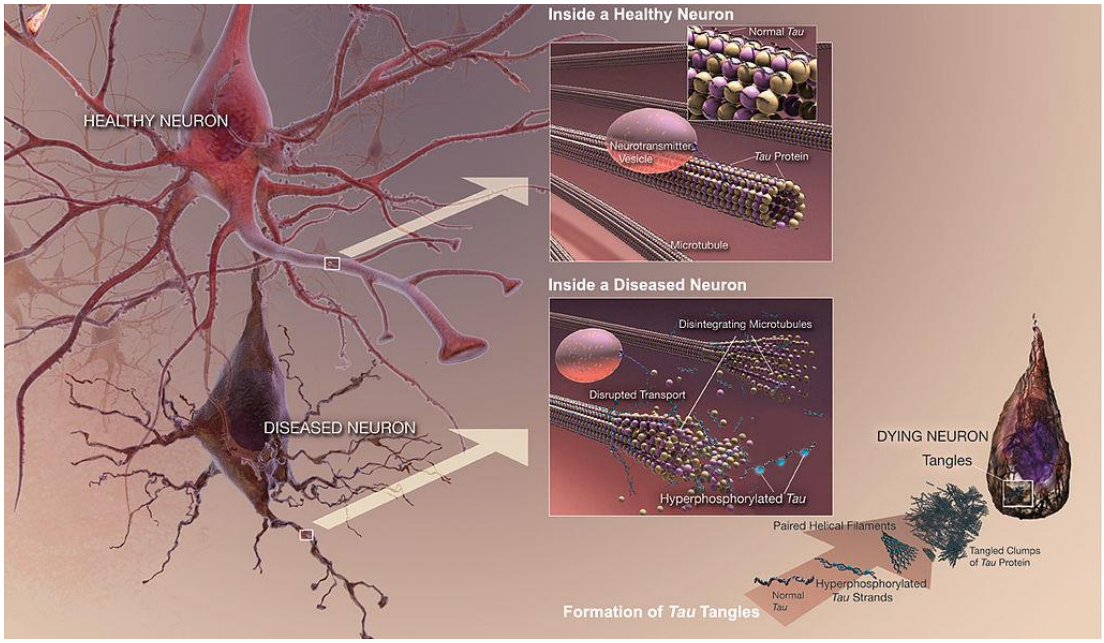


Fig. 2: Hyperphosphorylation of tau proteins leading to an unstable formation of microtubules.[7]

5.1.3 Neuroinflammation

Senile plaques are the source of infection and **inflammatory** response, which is promoted by fibrillation of Aβ.[1] Consequently immune cells like microglia, astroglia, pro-inflammatory cytokines (TNFα, IL-6, IL-1α, GMCSF), chemokines, caspases, complement system, nitric oxide and reactive oxygen species are activated.[8]

TNFα binds and transduces the signal by two TNF receptors: TNF Receptor 1 (TNFR1) and TNF Receptor 2 (TNFR2). TNFR1 causes damages through its pro-inflammatory effects. On the other hand, TNFR2 has neuroprotective properties. It is considered an activator of the NF-κB pathway, which leads to an anti-apoptic processes and thus it contributes to cell survival and neuronal protection.[1]

Selective inhibition of TNFR1 and/or activation of TNRF2 can possibly inhibit the neuroinflammatory pathway involved in neurodegenerative processes and utilize the neuroprotective effect of TNRF2.[1]

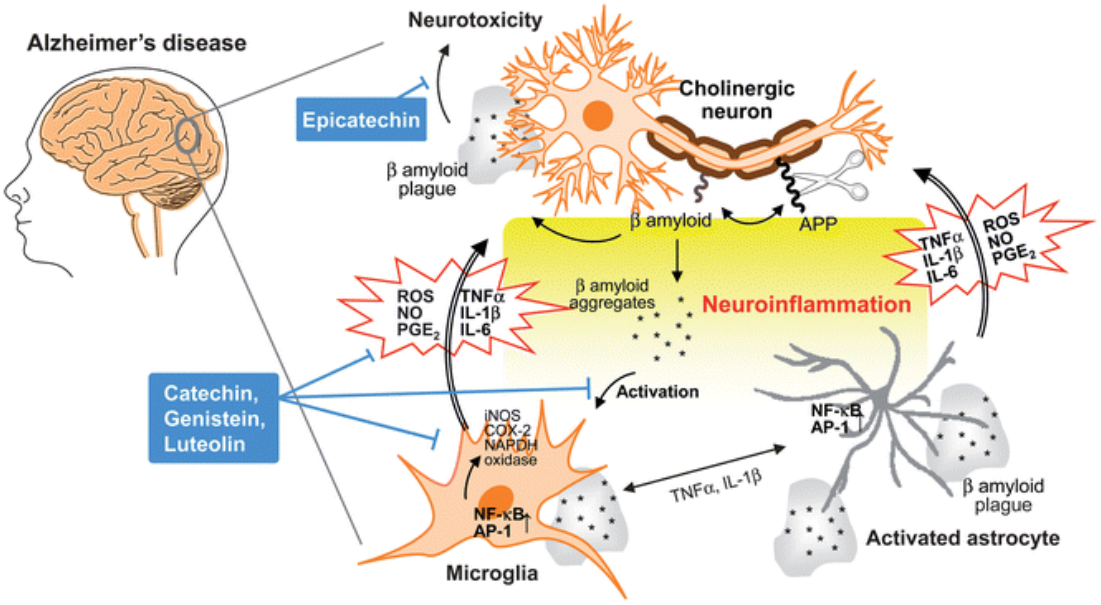


Fig. 3: Neuroinflammation and pro-inflammatory substances involved in the pathology of AD.[9]

5.1.4 Oxidative stress

Oxidative stress is an imbalance between production of reactive oxygen species (ROS) and antioxidant defence mechanisms that lead to accumulation of ROS.[10]

ROS are a group of reactive molecules that are derived from oxygen. ROS are usually short-lived and highly reactive because they contain an unpaired valence electron. Thanks to the electron they can react quickly with another radical or another molecule.[11,12] ROS are generated by exogenous and endogenous stimuli. Ultra violet light (UV) and ionizing radiation represent the exogenous source of ROS formation. Endogenous generation of ROS is mediated by mitochondrial and non-mitochondrial ROS-generating enzymes, e.g. nicotinamide adenine dinucleotide phosphate oxidase (NADPH), the major sources of ROS production, and further xanthine oxidase (XO), cytochrome P450 from endoplasmic reticulum and flavin oxidases from peroxisomes.[13]

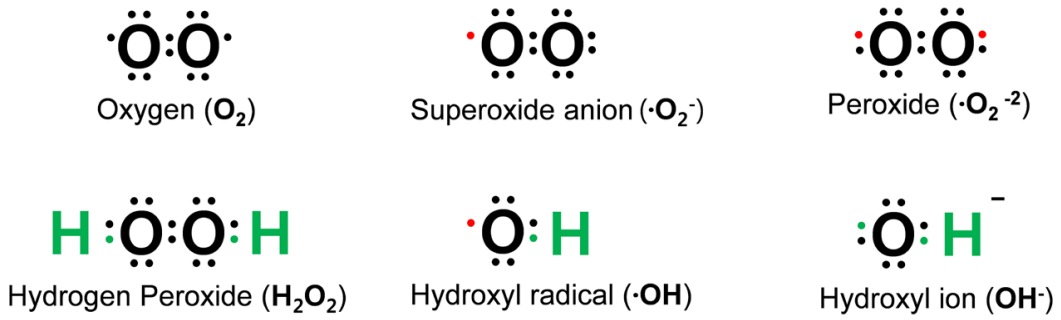


Fig. 4: Common reactive oxygen species.[12]

Although ROS are considered the molecules that affect organism negatively, they can promote physiological functions in cellular signalling related to the cardiovascular system, defence mechanisms of the immune system, regulation of mitosis, proliferation, migration, cell survival and apoptosis.[11,14,15] ROS can also affect the transcription factors, which regulate cellular response to ROS. Increased ROS concentration may cause activation of the antioxidant defence mechanisms. NF-E2 related factor 2 (Nrf2) is one of the redox-sensitive transcription factors. It is activated by ROS and it leads to an expression of antioxidant enzymes like superoxide dismutases, peroxiredoxins and glutathione peroxidases.[16] Nrf2 is normally present in cytoplasm thanks to a suppressor protein Kelch- like ECH- associated protein 1 (Keap1). This complex impedes the translocation of Nrf2 to the nucleus and maintains Nrf2 inactive under normal conditions. Higher ROS levels cleave the bond in this complex and Nrf2 activates gene transcription.[17]

Primarily, the formation of ROS is neutralized by several antioxidant systems mentioned before.

Due to the fact that the brain is one of the most metabolically active organs, it is very prone to oxidative stress, because of a high oxygen demand. The redox-active metals like iron or copper are involved in catalization and thus formation of ROS Another oxidative stress leading causes include high levels of polyunsaturated fatty acids that serve as substrates for lipid peroxidation, and low levels of glutathione (GSH), which is an endogenous antioxidant.[18,19]

This oxidative imbalance can lead to neuronal damage and can be crucial at the begging and progression of AD. The accumulation of Aβ probably induces oxidative stress and causes mitochondrial dysfunction and energy defeats. Oxidative stress can also stimulate hyperphosphorylation of protein tau, another factor that characterizes AD.[5,20]

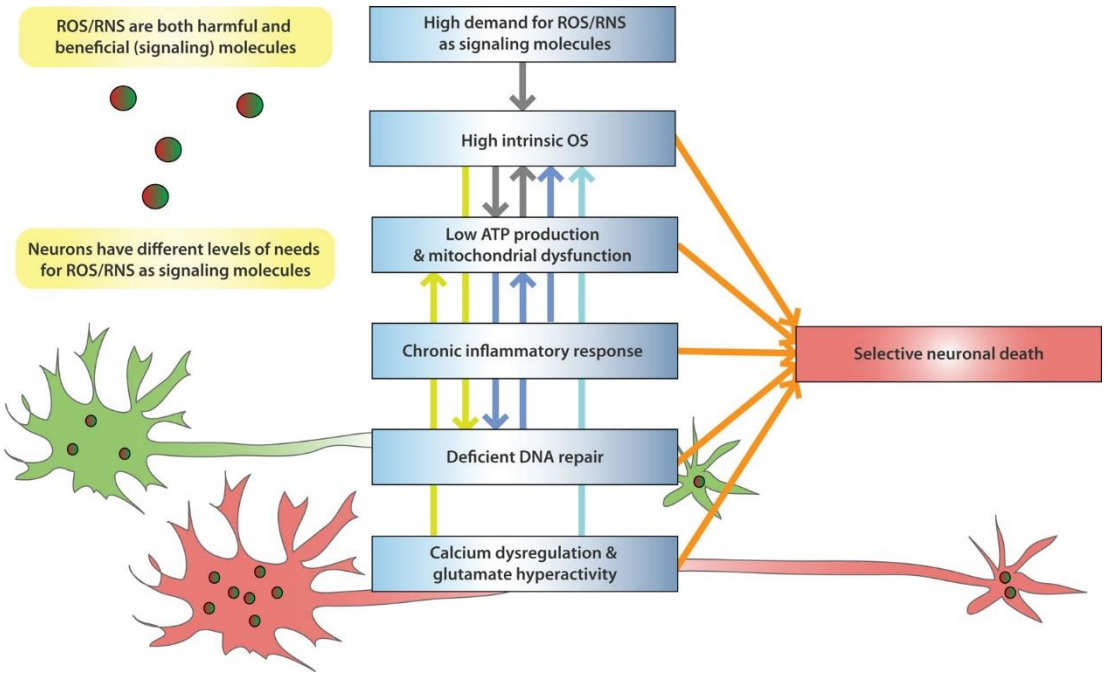


Fig. 5: Molecular and cellular factors, which contribute to the vulnerability of neurons to oxidative stress.[18]

5.2 Coumarins

Coumarins (2H-1-benzopyran-2-one) are heterocyclic compounds constituted of a benzene ring attached to an α-pyrone ring. More than 1300 coumarins have been identified as secondary metabolites of plants, bacteria and fungi. They can be isolated from the tonka bean (*Dipteryx odorata* Wild), and they are also present in several plant families like *Rutaceae, Caprifoliaceae, Oleaceae* and *Apiaceae*. Coumarins can be found in vegetables, fruits, seeds, nuts, coffee, tea and wine, but they can be prepared also synthetically. Coumarins are non-polar compounds with a short half-life, which leads to their low bioavailability. They are metabolized by cytochrome P-450-linked

mono-oxygenase (CYP 2A6) in liver, forming their primary metabolite 7-hydroxycoumarin- the active form of coumarins.[21]

The physicochemical properties and therapeutic applications of natural coumarins depend on the substitution of coumarin's structure.[22]

Coumarins have a wild range of biological effects including anti-inflammatory, antioxidant, neuroprotective, antibacterial, antifungal, antihypertensive, antitubercular, anticonvulsant, antiadipogenic, anticancer, antiviral and anticoagulant properties.[22]

The strong action of coumarins on the central nervous system seems to be a very important biological property, and, thus, they are a potential resource for the prevention and therapy of CNS diseases.[3]

Some studies showed that substitution at C7 of the coumarin nucleus with a small functional groups (e.g. hydroxyl, methoxy) had a lower inhibitory effect on AChE, while attaching bigger substituent increased the inhibitory activity of coumarins. Substitution at C3 modulated the AChE activity, as well as the activity of MAO-B. Positioning of methyl groups at C3 and C4 leads to formation of more active compounds towards both enzymes, while monosubstitution at position C4 with phenyl, trifluoromethyl, and hydroxyl groups lowered the MAO inhibitory activity.[23]

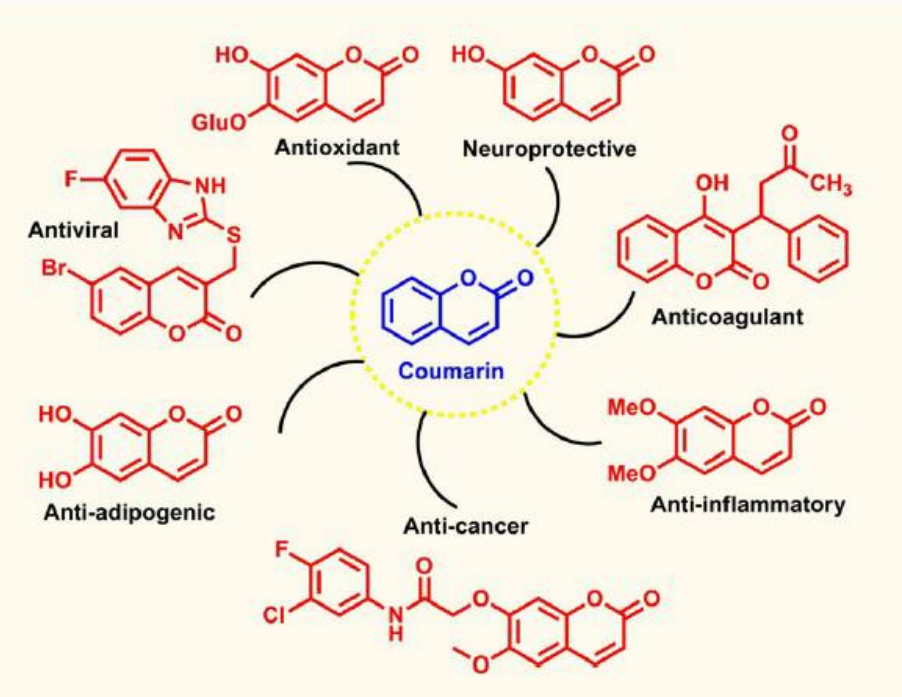
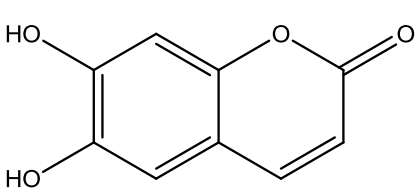
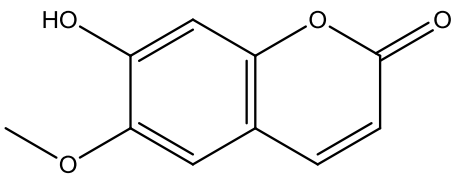


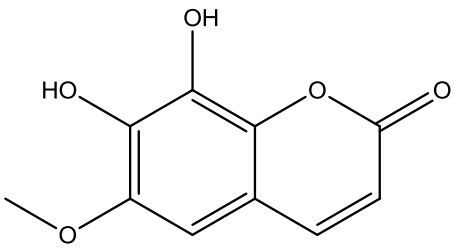
Fig. 6: Pharmacological properties of coumarins.[21]



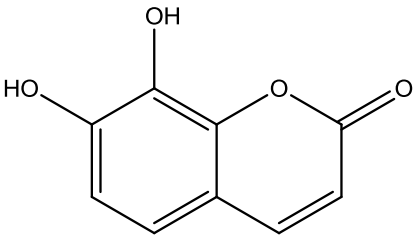
Esculetin



Scopoletin



Fraxetin



Daphnetin

Fig. 7: Chemical structures of tested series of coumarins.

Esculetin is a coumarin isolated from *Cichorium intybus* and *Bougainvilea spectabilis* Wild (*Nyctaginaceae*). Esculetin inhibits the cyclooxygenase and lipoxygenase enzymes, which explains its anti-inflammatory activity. It shows also anticancer, antioxidant and antiadipogenic properties and neuroprotective activity.[22]
It exhibits also an apoptotic action by decreasing the cleaved caspase-3 level, the up-regulation of the expression of Bcl-2, and the down-regulation of Bax expression that are two proteins related to apoptosis.[3]

Scopoletin was isolated from *Fraxinus rhynchophylla* (*Oleaceae*) and it shows antihypertensive and antiadipogenic features.[22]
Scopoletin and its glycoside scopolin were proved as potential AChE inhibitors. *In vitro* activity and *in vivo* experiments showed that both of these compounds increased the extracellular ACh concentration in the rat brain.[24]

Fraxetin is an ortho-dihydroxylated coumarin isolated from *Fraxinus rhynchophylla* (*Oleaceae*), which showed antiadipogenic properties.[22]

Daphnetin is a coumarin isolated from *Daphne papyracea* (*Thymelaeaceae*), which also determined antiadipogenic features.[22]

6 Aim of the thesis

The aim of this thesis was to evaluate neuroprotective effects of natural coumarins such as esculetin (ESC), daphnetin (DAPH), fraxetin (FRAX) and scopoletin (SCOP) using *in vitro* models.

Aβ-peptides were used to simulate neuroinflammation and neurodegeneration of SH-SY5Y cells.

7 Experimental part

Cell culture

Human neuronal–like SH–SY5Y cells of neuroblastoma were purchased from Lombardy and Emilia Romagna Experimental Zootechnic InstituteItaly.

Components of the culture medium

- Dulbecco's modified Eagle's medium (DMEM) - Lonza Group Ltd- Switzerland
- Stock solution of L - glutamine 200 mM - Sigma Aldrich Chemical St. Louis, MO, USA

  Prepared previously by dissolving 0,146 g of glutamine in 5 mL of distilled H2O and filtering with 0.22 μm filter of acetate cellulose.
- Stock solution of penicillin 5000 UI and 5 μg/mL streptomycin - Sigma Aldrich Chemical St. Louis, MO, USA
- Fetal bovine serum (FBS) - Lonza Group Ltd- Switzerland

  Previously deactivated at 57°C for 30 minutes.

Preparation of 10 % FBS medium

The medium was prepared into sterile containers under sterile conditions. Components were mixed in following order:

- DMEM 88%
- Penicillin-Streptomycin 1%
- L-glutamine 1%
- FBS 10%

Preparation of cell cultures

The cell culture was routinely grown in DMEM supplemented with 10 % FBS, 2 mM glutamine, stock solution of penicillin 5000 IU and 5 μg/mL streptomycin using 75 cm^2 cell tissue culture flasks and 37 °C humified incubator with 5 % CO_2,. When the

cell population reached confluence, EDTA-Trypsin (0.02-0.05%) solution was used for cell splitting (1:3 or 1:5 into new flasks every two weeks).

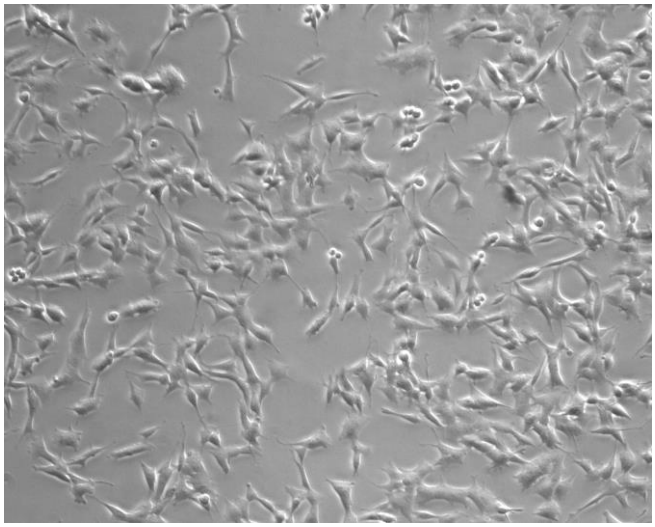


Fig. 8: SH-SY5Y cells (optical microscope, magnification 100x)

7.1 Materials and methods

Chemical compounds

- Esculetin, daphnetin, scopoletin and fraxetin were purchased from Sigma-Aldrich Chemical (St.Louis, Missouri, U.S.A.).
- Stock solution of esculetin 20 mM
  Prepared from 1 mL of sterile dimethyl sulfoxide (DMSO) and 3.562 mg of ESC
- Stock solution of daphnetin 40 mM
  Prepared from 1 mL of sterile DMSO and 7.1256 mg of DAPH
- Stock solution of scopoletin 40 mM
  Prepared from 1 mL of sterile DMSO and 7.6868 mg of SCOP
- Stock solution of fraxetin 40 mM
  Prepared from 1 mL of sterile DMSO and 8.3268 mg of FRAX

Solutions of these coumarins were aliquoted into an eppendorf of 1.5 mL and stored at -20 °C. Working solutions were prepared by diluting the stock solution in a culture medium or in Hank's balanced salt solution (HBSS).

- β-amyloid peptide 1-42 ($A\beta_{1-42}$) was purchased from AnaSpec (Freemont, CA, USA).

Preparation of peptide

Peptides were subjected to the denaturation: 1 mg of $A\beta_{1–42}$ was dissolved in 500 μL of 1,1,1,3,3,3-hexafluoro-2-propanol and sonicated for 1 minute at average intensity. Prepared solution was consequently sealed with parafilm and left to dissolve for 24 hours. Subsequently, the solvent was allowed to completely evaporate by leaving the peptide solution in a laminar flow for 24 hours. The obtained peptide was utilized for preparation of stock solutions of 1 mM $A\beta_{1-42}$ in sterile DMSO. Stock solutions were poured into vials (0.5 mL aliquots) and stored at -20 °C.

Preparation of oligomers

240 μL of DMEM were added to 10 μL of $A\beta_{1-42}$ and this mixture was stored at 4 °C for 24 hours to allow formation of oligomers ($OA\beta_{1-42}$).

3.4.2 Solutions

Hank's balanced salt solution (HBSS) without phenol red (Lonza).

Dulbecco's phosphate buffered saline without Ca^{2+} and Mg^{2+} 10x (D'PBS) (Lonza).

Dulbecco's phosphate buffered saline with Ca^{2+} and Mg^{2+} 10x (D'PBS) (Lonza).

0.02 – 0.05 % EDTA-trypsin solution (Lonza) diluted by sterile D'PBS 1:10

0.25 % eosin solution, prepared from appropriate amount of Eosin B (Sigma Aldrich) and D'PBS.

Stock solution of salts of tetrazolium: 30 mg of salts of tetrazolium (MTT) (Sigma Aldrich) were dissolved in 6 ml of HBSS and the solution was stored at -20°C protected from light.

Experimental solution of MTT was prepared from 1.2 mL of stock solution of MTT, which was added into 10.8 mL of HBSS. For the preparation, 0.45 μm filters were used and the solutions were prepared in the time of need and protected from light.

Stock solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was prepared from 3,94 mg of DPPH (Sigma Aldrich) dissolved in 1 mL of ethanol (EtOH). The solution was stored at -20°C and protected from light.

Experimental solution of 100μM DPPH was prepared from 180 μL of DPPH, which was added into 17.820 μL of EtOH.

Experimental solution of t-BuOOH (400 μM) was prepared from an original solution of t-BuOOH (Sigma Aldrich Chemical St. Louis, MO, USA). 50 μL of original solution were added to 420 μL of HBSS to prepare a stock solution. Consequently, 10 μL of stock solution and 990 μL of HBSS were mixed to prepare an intermediate solution with the concentration of 10 mM. According to a calculation and consideration of 12 mL of working solution for 96 well plate, 480 μL of intermediate solution and 11.52 mL of HBSS were mixed

Stock solution of 2',7'-dichlorohydrofluorescein diacetate (H_2DCF-DA) was prepared from 2.5 mg of H_2DCF-DA (Sigma Aldrich) dissolved in 1 mL of dimethyl sulfoxide (DMSO). The solution was stored at -20°C protected from light.

10 µg/mL experimental solution of H_2DCF-DA was prepared from 50 µL of H_2DCF-DA stock solution, which was added into 12.450 µL of HBSS.

Stock solution of monochlorobimane (MCB) (Sigma Aldrich) was prepared from 25 mg of MCB dissolved in 2 ml of 95% EtOH. The solution was stored -20°C protected from light.

50 µM experimental solution of MCB was prepared from 22 µl of MCB stock solution, which was added into 24.178 µl of D'PBS. The solution was prepared at the time of experiment and protected from light.

7.2 Experiments

7.2.1 Evaluation of intrinsic antioxidant activity

Principle of the assay

DPPH is an organic chemical compound, 2,2-diphenyl-1-picrylhydrazyl. It is a blue-dark-colored crystalline molecule with an unpaired electron. DPPH is a well-known radical and a trap ("scavenger") for other radicals. Therefore, a level of DPPH reduction is utilized as an indicator of the radical nature of tested molecules. By reacting the DPPH with an antioxidant compound capable of reducing the radical compound, a dark blue discoloration of the solution occurs due to the disappearance of the radical and a solution becomes yellow. This colour changes allow visual monitoring of this neutralization reaction, and the amount of trapped radicals can be expressed due to the change in optical absorption.[25,26]

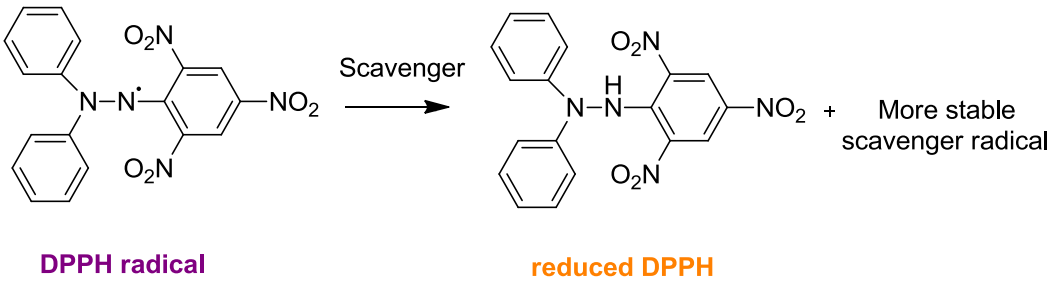


Fig. 9: The scheme of DPPH neutralization.

Measurement

The suspension of SH-SY5Y cells at density of 2.10^5 cells/mL in 10 % FBS medium was prepared. Consequently, 100 µL of suspension were added into black wells of the 96-well plate, while no cells were added into white wells. The well plate was incubated for 24 hours at 37 °C and 5% of CO_2 to allow a formation of monolayer.

To evaluate the intrinsic antioxidant activity of the compounds, the standard of 100 µM DPPH was used.

The amount of 50 μL of ESC, SCOP, FRAX and DAPH solutions of certain concentrations (2.5, 5, 10, 20, 40 μM), dissolved in EtOH, were dispensed in a 96-well plate. At least three repetitions of the experiment were made for higher accuracy.
Further, 150 μL of 100 μM experimental solution of DPPH were added into each well and 200 μL in wells of negative control. The 96-well plate was further incubated for 30 minutes at room temperature protected from light. After incubation, the discoloration was quantified through the measurement of absorbance at $\lambda = 492$ nm by a multilabel plate reader (VICTOR^{TM} X3, PerkinElmer, Massachusetts, USA).

Expression of results

The results were expressed as a percentual inhibition of DPPH radical with coumarin compounds in comparison to a control sample. The percentage was calculated using the following formula:

$$\% \ inhibition \ of \ DPPH = \frac{ODdpph - ODcoumarins}{ODdpph}$$

ODdpph – ODcoumarins = Optical density of the solutions formed by DPPH and coumarins
ODdpph = Optical density of the DPPH solution

7.2.2 Determination of ROS intracellular formation

Principle of the assay

For this experiment, t-BuOOH was utilized as an exogenous inductor of oxidative stress. This organic peroxide is usually used as a model substance for evaluation of mechanisms of cellular alterations resulting from oxidative stress in cells.

The intracellular ROS formation was evaluated thanks to 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA), a non-polar and non-fluorescent molecule, which is able to diffuse rapidly into the living cells. Consequently, the H_2DCF-DA is hydrolysed by intracellular esterase to 2',7'-dichlorodihydrifluorescein (H_2DCF). The H_2DCF is a polar molecule, which remains trapped in the cytoplasm, where it becomes oxidized because of a presence of ROS. As a result a fluorescent molecule, 2',7'-dichlorofluorescein (DCF), is formed. The fluorescence intensity is directly proportional to the level of DCF created in cells, und thereby indirectly also to the ROS concentration.[27]

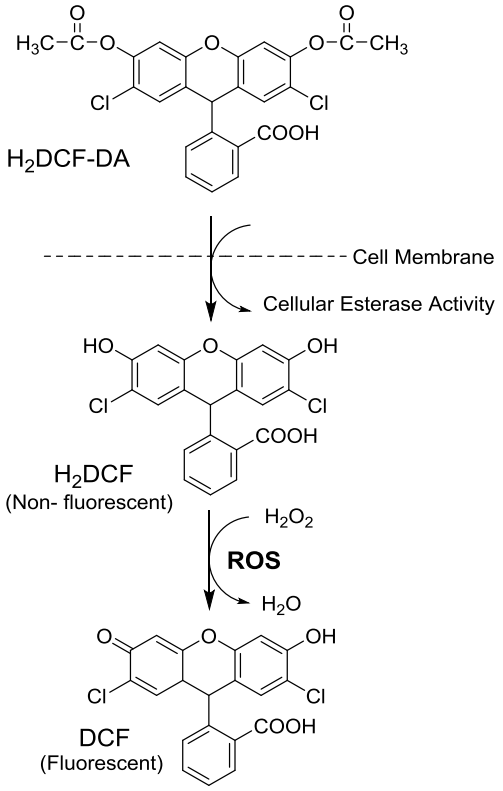


Fig. 10: The scheme of intracellular ROS concentration evaluation.

Measurement

A cellular suspension of SH-SY5Y with a density of $3x10^5$ cell/mL was prepared in 10% FBS medium. Consequently, 100 µL of suspension were spread into a 96-well plate and it was inserted into an incubator for 24 hours in a condition of 37 °C and 5% of CO_2 to allow adhering to the surface.

The cells were treated by adding 100 µL of DMEM with different concentrations of ESC, SCOP, FRAX and DAPH; while in the negative controls, it was added 100 µL of DMEM. Further, cells were incubated in the same conditions as mentioned before for 2 and 24 hours. At least three repetitions of the experiment were made for higher accuracy.

The content of the plate was removed with an inversion of a plate and each well was washed with 150 µL of HBSS, then 100 µL of experimental solution of H_2DCF-DA were added into each well. The well plate was further incubated for 30 minutes at room temperature protected from light. After incubation, the solution of H_2DCF-DA was eliminated by the inversion of a plate and the cells were washed with 150 µL of HBSS. 200 µL of HBSS with 200 µM t-BOOH were added to each tested plate. 100 µL of HBSS with 200 µM t-BOOH and 100 µL of pure HBSS were added into positive controls, while 200 µL of HBSS were added into the negative controls.

After 30 minutes, the fluorescence measurement was performed by flow cytometry (VICTOR^{TM}, PerkinElmer, Massachusetts, USA) at $\lambda_{excitation}$ = 485 nm and $\lambda_{emission}$ = 535 nm.

Expression of results

The intracellular formation of ROS was expressed as the increase of fluorescence using this formula:

$$Fluorescence\ increase = \frac{Fc}{Fnc}$$

Fc = fluorescence of cells treated with t-BuOOH and with different concentrations of coumarins

Fnc = fluorescence of untreated cells

7.2.3 Determination of intracellular glutathione levels

Principle of the assay

Glutathione (GSH) is a tripeptide synthesized from glutamate, cysteine and glycine. GSH is one of the most important antioxidants, which protects cells against oxidative stress. In the brain, the enzyme glutamylcysteine synthetase produces GSH utilizing glutamate and cysteine as substrates. This dipeptide is then associated with glycine by the catalization of glutathione synthetase to form a complete GSH. Several studies have also demonstrated that GSH is involved in inhibiting apoptic cell death and DNA damage. Reduced GSH contains a free thiol group and reacts non-enzymatically with ROS, which leads to their removal. This reaction promotes a formation of glutathione disulfide (GSSG), which is the final product of GPX reactions. Reduced GSH can be regenerated from glutathione disulfide by the reaction with glutathione reductase within the transfer of electrons from NADPH to glutathione disulfide.[28]

When cells are exposed to increased levels of oxidative stress, GSSG accumulates and the ratio of GSH to GSSG decreases. Therefore, the determination of the GSH/GSSG ratio and the quantification of GSSG are useful indicators of oxidative stress in cells and tissues. Higher levels of GSH indicate bigger resistance of SH-SY5Y cells to neuronal death, in terms of apoptosis.[28]

To measure the intracellular glutathione (GSH) formation, monochlorobimane was used (MCB, Fluorescent Dyes, Los Angeles, Ca, U.S.A). This MCB probe is specific to SH group of glutathione and they form a fluorescent complex together.

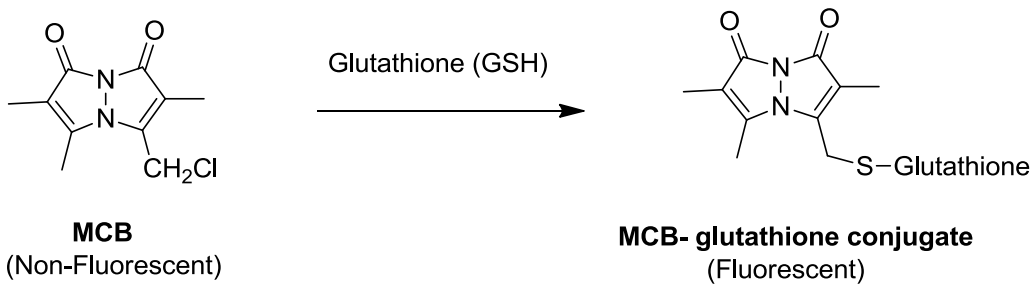


Fig. 11: Formation of fluorescent complex of GSH with MCB.

Measurement

The suspension of SH-SY5Y cells at density of 2 x 10^5 cells/mL in 10 % FBS medium was prepared. Consequently, 100 μL of suspension were added into black wells of the 96-well plate, while no cells were added into white wells. The well plate was incubated for 24 hours at 37 °C and 5% of CO_2 to allow a formation of monolayer.

The content of the plate was eliminated and SH-SY5Y cells were treated with 100 μL of DMEM with ESC (10 μM, 20 μM, 40 μM), while in the negative controls, it was added 100 μL of DMEM. Further, cells were treated with oligomeric $A\beta_{1-42}$ (2 μM) (pre-treatment).

For the second experiment, cells were treated with esculetin (10 μM, 20 μM, 40 μM) and oligomeric $A\beta_{1-42}$ (2 μM) (co-treatment) all at once.

Cells were consequently incubated at the same conditions as before for 24 hours. At least three repetitions of the experiment were made for higher accuracy.

At the end of the treatment, the medium was eliminated from the wells and the cells were incubated with 100 μL of MCB (50 μM) for 30 minutes protected from light. The GSH levels were measured by a multilabel plate reader (VICTORTM X3) at $\lambda_{excitation}$ = 360 nm and $\lambda_{emission}$ = 465 nm.

Expression of results

The increase of fluorescence was calculated using the following formula:

$$\text{Fluorescence increase} = \frac{Fi}{Fni}$$

Fi = Fluorescence of the cells treated with coumarins, after subtracting the control

Fni = Fluorescence of the control solution

7.2.4 Determination of neuronal viability

Principle of the assay

The MTT assay is a colorimetric method for evaluating neuronal viability. Oxidoreductase enzymes like mitochondrial NAD(P)H reduce a yellow tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble form of formazan, which has a purple colour. Reduction of MTT depends on the neuronal activity due to level of NAD(P)H. Neurons with low activity reduce MTT hardly at all.[27]

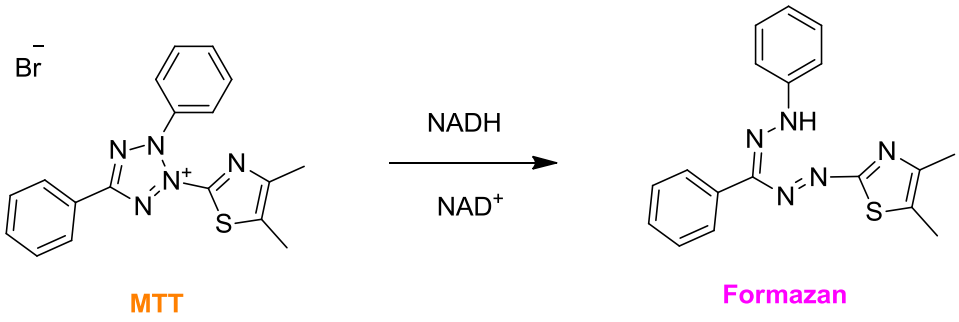


Fig. 12: Formation of formazan thanks to the activity of vital cells.

Measurement

A cellular suspension of SH-SY5Y was prepared with a density 2 x 10^5 cell/mL in 10% FBS medium. Consequently, 100 µL of suspension were spread into a 96-well plate and it was inserted into incubator for 24 hours in a condition of 37°C and 5% of CO_2 to allow a formation of monolayer.

To assess the neurotoxicity of coumarin compounds, the cells were treated by adding 100 µL of DMEM with ESC, SCOP, FRAX and DAPH (2.5-80 µM); while in the negative controls, it was added 100 µL of DMEM. Further, cells were incubated for 4 hours in the same conditions. At least three repetitions of the experiment were made for higher accuracy.

To perform the test, the content of the plate was removed and 100 µL of MTT experimental solution were added into each well. After 24 hours of incubation, the

solution of tetrazolium salts was eliminated. After removal of MTT, the formazan crystals were washed with 150 µL of HBSS and further they were dissolved in 150 µL of isopropanol to extract and solubilize the precipitates of formazan. To facilitate the solubilisation process the plates were subjected to agitation to obtain a homogeneous solution. The amount of formazan was measured at λ = 570 nm (ref. λ = 690 nm) with a spectrophotometer (VICTOR^{TM} X3, PerkinElmer, Massachusetts, USA).

Expression of results

Relative cell viability was expressed as percentage compared to control cells and calculated using this formula:

$$Relative\ cell\ viability = \frac{ODt}{ODtn}.100$$

ODt = Absorbance of the solution with treated neurons

ODtn = Absorbance of the solution with untreated neurons

7.2.5 Determination of neuroprotective effects

Principle of the assay

The amyloid beta peptide (1-42) induced cytotoxicity, in terms of exocytosis and intracellular reduction of formazan was determined with the use of tetrazolium salts.

Measurement

A cellular suspension of SH-SY5Y was prepared with a density 3 x 10^5 cell/mL in 10% FBS medium. Consequently, 100 µL of suspension were spread into a 96-well plate and it was inserted into incubator for 24 hours in a condition of 37°C and 5% of CO_2 to allow a formation of monolayer.

To assess the neuroprotective effects of coumarins, the cells were treated by adding 50 µL of DMEM 2% FBS with ESC (20 µM) and 50 µL of DMEM 2% FBS with $OA\beta_{1-42}$ (10µM); in the positive control, it was added 50 µL of DMEM 2% FBS with ESC (20 µM) or 50 µL of DMEM 2% FBS with $OA\beta_{1-42}$ (10µM) and 50 µL of DMEM 2% FBS; while in the negative controls, it was added 100 µL of DMEM 2% FBS. Further, cells were incubated for 4 hours in the same conditions. At least three repetitions of the experiment were made for higher accuracy.

To perform the test, the content of the plate was removed and 100 µL of MTT experimental solution were added into each well. After 1 hour of incubation, 10 µL of 10% v/v tween-20 were added for allow the solubilisation of intracellular granules of formazan (TS-MTT). To facilitate the solubilisation process of intracellular granules of formazan the 96-well plate was subjected to agitation at 300 rpm for 10 minutes at 37°C. Then the entire volume of every well was transferred to a new 96-well plate and the amount of intracellular formazan was measured at λ = 570 nm (ref. λ = 690 nm) with a spectrophotometer (VICTOR™ X3, PerkinElmer, Massachusetts, USA).

Subsequently solutions were carefully aspirated from all the wells using a multichannel pipette and further exocytosed crystals of formazan (TI-MTT) were dissolved in 100 µL of isopropanol. To facilitate the solubilisation process the 96-well plate were subjected to agitation to obtain a homogeneous solution. The amount of

exocitosed crystals of formazan was measured at λ = 570 nm (ref. λ = 690 nm) with a spectrophotometer (VICTOR^{TM} X3, PerkinElmer, Massachusetts, USA).

Expression of results

Relative amyloid beta peptide (1-42) induced formazan exocytosis was expressed as percentage compared to control cells and calculated using this formula:

$$Formazan\ exocytosis = \frac{ODt}{ODtn}.100$$

ODt = Absorbance of the solution with treated neurons

ODtn = Absorbance of the solution with untreated neurons

Statistical analysis

The obtained experimental data were analysed using t-test and GraphPad Prism software, version 3.03. Values of $P \leq 0.05$ were considered statistically significant.

8 Results

Neurotoxicity

Initially, we determined the neurotoxicity of ESC, SCOP, FRAX and DAPH. SH-SY5Y cells were incubated for 24 hours with various concentrations of the studied compounds (2.5-80μM). At the end of incubation, the neuronal viability was measured using MTT assay as previously described. The results are expressed as percentage compared to control cells.

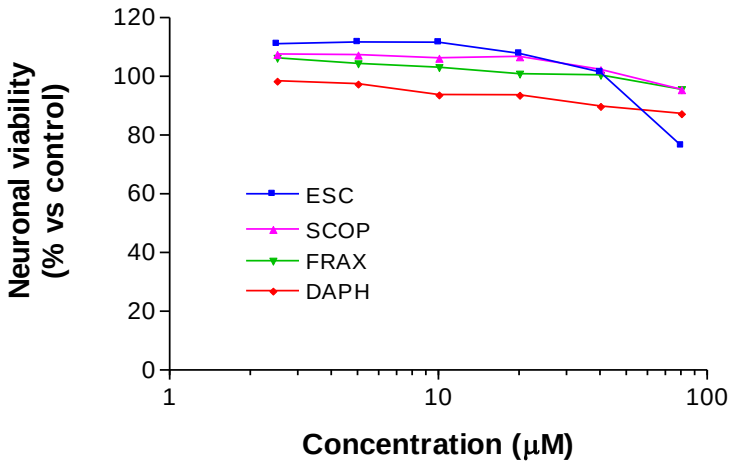


Fig.13: Neuronal viability in SHSY5Y cells after 24 hours of treatment with different concentration of ESC, SCOP, FRAX and DAPH. The results refer to at least three independent experiments.

The treatment of SH-SY5Y cells with ESC, SCOP, FRAX and DAPH concentrations up to 40 μM did not affect neuronal viability. Therefore, we selected the range of nontoxic concentrations 2.5-40 μM for all the subsequent experiments.

Intrinsic antioxidant activity

To evaluate the intrinsic antioxidant activity of ESC, SCOP, FRAX and DAPH we used the DPPH assay. The discoloration was quantified through the measurement of decrease in absorbance at 492 nm and it was directly proportional to the antioxidant activity.

We recorded that ESC, FRAX and DAPH possess significant scavenging properties against DPPH. ESC shows the strongest quenching capacity against DPPH, followed by DAPH. While FRAX is less active than ESC and DAPH in DPPH scavenging. SCOP is not effective against DPPH. DPPH radical scavenging was determined by the described method.

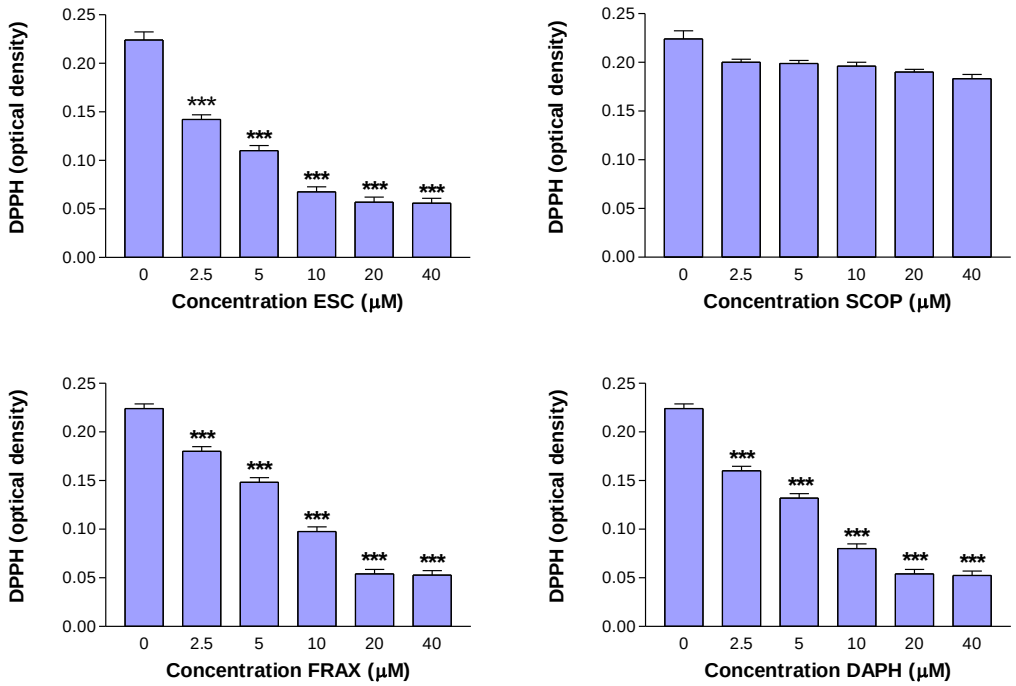


Fig. 14: Antioxidant direct activity of ESC, SCOP, FRAX and DAPH. The results are expressed as mean ± SD of at least three independent experiments (*** $p < 0.001$ vs control; t-test).

ROS formation inhibition

Subsequently, we evaluated the antioxidant activity of ESC, SCOP, FRAX and DAPH in SHSY5Y cells against the intracellular t-BuOOH-induced ROS formation. SH-SY5Y cells were incubated 2 hours with various concentrations of the studied coumarins and then treated with t-BuOOH (200 µM) for 30 minutes, in order to allow the entrance of the coumarins in SHSY5Y cells before the oxidative treatment.

We recorded that ESC, FRAX and DAPH (2.5-40 µM) counteract t-BuOOH-induced ROS formation in SH-SY5Y cells. While we did not observe antioxidant effects for SCOP.

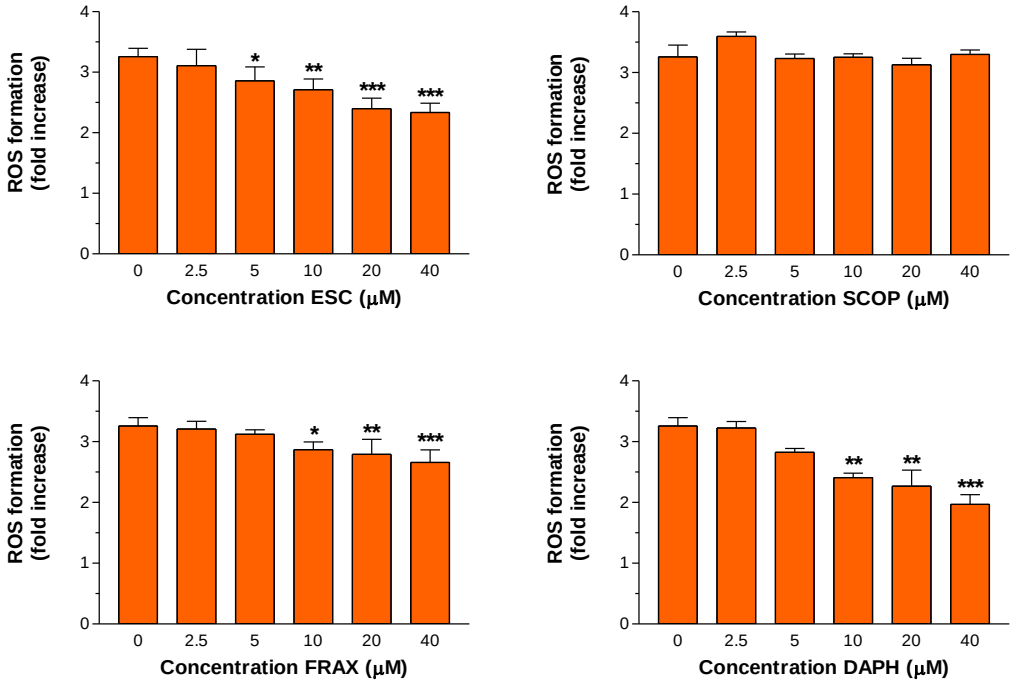


Fig. 15: ROS formation in SHSY5Y cells after 2 hours of treatment with different concentrations of ESC, SCOP, FRAX and DAPH and 30 minutes of treatment with t-BuOOH (200 µM). The results are expressed as mean ± SD of at least three independent experiments (*p<0.05 vs treated with t-BuOOH; **p<0.01 vs treated with t-BuOOH; ***p<0.001 vs treated with t-BuOOH; t-test).

SH-SY5Y cells were therefore incubated for 24 hours with various concentrations of the studied coumarins (2.5-40µM) and then treated with t-BuOOH (200 µM) for 30 minutes. At the end of incubation, the ROS formation was determined using a fluorescent probe, H_2DCF-DA, as previously described.

We observed that ESC and DAPH prevent the t-BuOOH ROS formation in SHSY5Y cells.

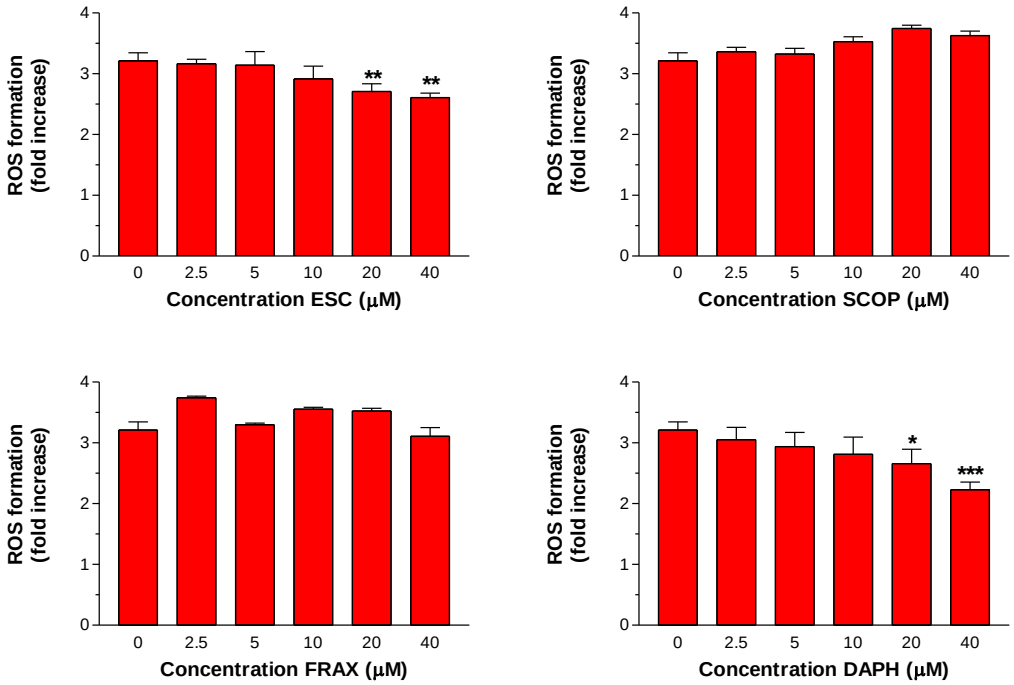


Fig. 16: ROS formation in SHSY5Y cells after 24 hours of treatment with different concentrations of ESC, SCOP, FRAX and DAPH and 30 minutes of treatment with t-BuOOH (200 µM). The results are expressed as mean ± SD of at least three independent experiments (*p<0.05 vs treated with t-BuOOH; **p<0.01 vs treated with t-BuOOH; ***p<0.001 vs treated with t-BuOOH; t-test).

Intracellular GSH levels increase

Then we evaluated the ability of ESC to increase the intracellular GSH levels. SH-SY5Y cells were incubated for 24 hours with various concentrations of ESC (10-40 µM). At the end of incubation, GSH levels were measured using a fluorescent probe, monochlorobimane, as previously described. We recorded that ESC enhances the total GSH levels of SH-SY5Y cells.

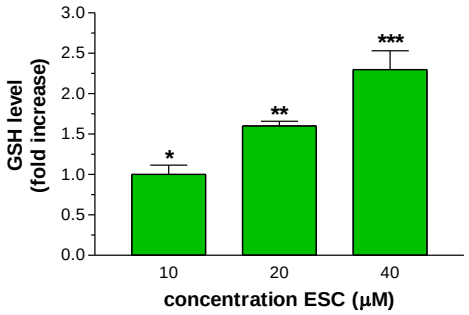


Fig. 17: Intracellular GSH levels in SHSY5Y cells after 24 hours of treatment with various concentration of ESC (10-40 µM). The results are expressed as mean ± SD of at least three independent experiments (*p<0.05 vs control; **p<0.01 vs control; ***p<0.001 vs control; t-test).

Neuroprotection

Finally, to evaluate the neuroprotective effects of ESC, SH-SY5Y cells were incubated for 4 hours with ESC (20µM) and $OA\beta_{1-42}$ (10µM). We observed that ESC is able to reduce the Aβ induced toxicity, in terms of exocytosis of formazan. At the end of incubation, the ability of ESC to counteract early phase of $OA\beta_{1-42}$-induced neurotoxicity was measured by MTT formazan exocytosis.

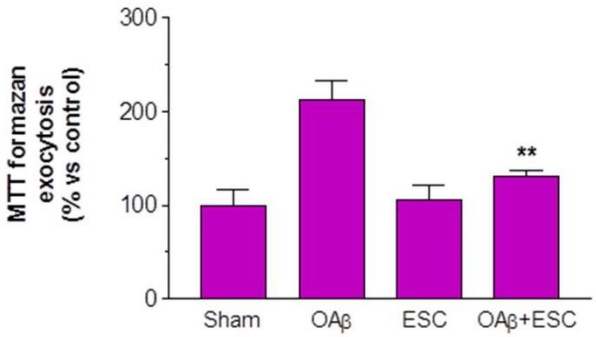


Fig. 18: Exocytosed formazan levels in SHSY-5Y after 4 hours of treatment with $OA\beta_{1-42}$ (10µM) and ESC (20 µM). The results are expressed as mean ± SD of at least three independent experiments (**p<0.01 vs treated with OAβ; t-test).

Sham term is used for a control sample with untreated cells.

9 Discussion

Although the etiology of AD remains unknown, the neuropathological profile is associated with memory loss and the presence of numerous plaques and cholinergic deficiency because of the degeneration or atrophy of cholinergic neurons in the basal forebrain.[29] Patients affected by AD exhibit abnormal deposits of Aβ, formation of neurofibrillary tangles, increased oxidative stress, and low levels of Ach.[30]

Unfortunately, there is no cure for AD, and treatment strategies are especially symptomatic.

To evaluate the potential of coumarins as anti-AD agents, we investigated their antioxidant and neuroprotective effects. Using MTT cytotoxicity assay, we could select four coumarine compounds in a range of certain concentrations (2.5 and 40 μM) that is not associated with neurotoxicity and could be applied in the subsequent studies.

We observed that OAβ1-42 dramatically enhanced MTT formazan exocytosis, resulting in the inhibition of cellular MTT reduction. Basal MTT formazan exocytosis and amyloid peptide-enhanced MTT formazan exocytosis were blocked by **ESC at the concentration of 20 μM**. According to the obtained results, we could say that among the tested coumarins ESC possess a direct neuroprotective effect against both t-BOOH-induced oxidative stress and OAβ1-42 induced neurotoxicity.

The observed direct cytoprotective action could be ascribed to numerous complex mechanisms such as the ability of ESC to prevent the bond between Aβ oligomers and membrane. Probably, ESC interacts directly with specific amino acids of amyloid peptide through the hydrogen bonds. These amino acids are important for the β amyloid aggregation, its bond with the membrane and the subsequent cytotoxicity.[31]

The antioxidant activity of the tested coumarins observed in this study correlate with the data reported in the scientific literature.[32]

Kim *et al*. [33]also tested the protective effect of ESC via scavenging reactive oxygen species. The radical scavenging activity was determined by DPPH radical, hydroxyl radical, and ROS. In addition, lipid peroxidation, ELISA test, Western blotting and immunofluorescence images were utilized for the measurement. In this study, ESC exhibited DPPH radical scavenging, hydroxyl radical scavenging, and intracellular

ROS scavenging activities. The scavenging activity of ESC resulted in the protection of cells from lipid peroxidation, protein carbonyl, and DNA damage induced by H_2O_2. Further, ESC recovered cell viability exposed to H_2O_2.[33]

Moreover, in another study, Ali *et al*.[32] confirmed the activity of coumarins isolated from *A. decursiva* and *A. capillaris* against AChE, BChE and BACE1, which further favour the use of coumarines like ESC and DAPH as potential treatment approaches for AD. They used the colorimetric determination of acetylcholinesterase activity to measure inhibitory activities of isolated coumarins towards AChE, BChE and BACE1. The inhibitory activity of ESC and DAPH towards AChE with IC_{50} 6.13 abd 11.57 μM were good. In addition, BACE1 inhibitory activity was strong having IC_{50} 7.67 and 11.19 μM. ESC and DAPH showed also a good inhibition activity towards BChE, concretely with IC_{50} 9.29 and 8.66 μM. To follow the results of this study, they performed molecular docking simulations using Autodock 4.2. They simulated interactions between BACE1 and coumarins and they confirmed that ESC and DAPH could prevent AD by targeting the formation of β-amyloid. Coumarins interact with the BACE1 through hydrogen bonds. This fact showed that hydrogen bonding and carboxylic groups might result in more effective enzyme inhibition.[32] Coumarins, which have a catechol group such as ESC, FRAX and DAPH, possess significant free radical scavenging activity and inhibitory effects on lipid peroxidation.[34] Substitution with a methoxyl or glycosyl group probably reduces the antioxidant activity like in the case of scopoletin.[32]

The relationship between structure and the activity could explain the results of this thesis that confirmed that ESC is a perspective compound for the treatment of AD. It has the highest antioxidant and cytoprotective capacity of tested coumarins. The structure of esculetin contains hydroxyl groups, which can interact easily with the specific part of enzymes like AChE, BChE and BACE1, which are involved in the pathogenesis of AD.

DAPH, which also contains hydroxyl groups showed good scavenging activity against DPPH. DAPH also determined its antioxidant capacity towards ROS formation. Although, DAPH had good antioxidant and scavenging profile, ESC showed the best

properties and thus we focused on the tests performed with ESC. The lower effect of FRAX and SCOP could be explained by their chemical structures.

In addition, 20µg of ESC were administered intracerebroventricularly into mice.[35] In this *in vivo* study, they investigated the neuroprotective effect of ESC on cerebral ischemia. ESC significantly reduced neurological deficit after 75 minutes of ischemia and 24 hours of reperfusion. ESC also demonstrated its post neuroprotective effect when administered after 4 hours of reperfusion. In this study, ESC also determined the properties mentioned in 5.2.[3,35]

Our results coincide with the results of studied mentioned before. ESC is the most tested compound of coumarins, because of its great results and promising utilization.

Results acquired in this research could contribute to a new possible treatment of AD with high perspectivity, lower side effects and good patient's compliance.

10 Conclusion

This experimental thesis was focused on neuroprotective and antioxidant properties of natural coumarins - ESC, DAPH, SCOP, and FRAX.

The results highlight that ESC possess significant neuroprotective effects compared to the other coumarins. In particular, our results showed the ability of ESC to prevent and counteract t-BOOH- and OAβ-induced neurotoxicity in SH-SY5Y cells.

Our study promotes further *in vivo* evaluation of ESC effect in the treatment of Alzheimer using relevant animal models and evaluate the potential therapeutic profile of ESC as a novel neuroprotective agent. Moreover, ESC could represent a privileged scaffold for the design and development of new antineurodegenerative drugs.

11 List of references

[1] VAN DIJK G., VAN HEIJNINGEN S., REIJNE A. C., NYAKAS C., VAN DER ZEE E. A. and EISEL U. L. M.: Integrative neurobiology of metabolic diseases, neuroinflammation, and neurodegeneration. *Frontiers in Neuroscience,* 2015, 9 (173), ISSN 1662-453X. DOI 10.3389/fnins.2015.00173.

[2] ALI M. Y., JANNAT S., JUNG H. A., CHOI R. J., ROY A. and CHOI J. S.: Anti-Alzheimer's disease potential of coumarins from Angelica decursiva and Artemisia capillaris and structure-activity analysis. *Asian Pacific Journal of tropical medicine,* 2016, 9 (2), p103-111. ISSN 1995-7645. DOI 10.1016/j.apjtm.2016.01.014.

[3] SKALICKA-WOŹNIAK K., ORHAN I. E., CORDELL G. A., NABAVI S. M. and BUDZYŃSKA B.: Implication of coumarins towards central nervous system disorders. *Pharmacological Research*, 2016, 103, p188-203. ISSN 1043-6618. DOI 10.1016/j.phrs.2015.11.023.

[4] SCHULZ V.: Ginkgo extract or cholinesterase inhibitors in patients with dementia: What clinical trials and guidelines fail to consider. *Phytomedicine*, 2003, 4 (4), p74-79. ISSN 0944-7113. DOI 10.1078/1433-187X-00302.

[5] MATTSON M.P.: Pathways towards and away from Alzheimer's disease. *Nature,* 2004, 430 (7000), p631-639. ISSN 0028-0836. DOI 10.1038/nature02621.

[6] SCHONROCK N., MATAMALES M., ITTNER L.M. and GÖTZ J.: MicroRNA networks surrounding APP and amyloid-β metabolism-implications for Alzheimer's disease. *Experimental neurology*, 2012, 235 (2), p447-454. ISSN 0014-4886. DOI 10.1016/j.expneurol.2011.11.013.

[7] U.S. Department of Health and Human Services. Publication Number: 08-3782. September 2008.
http://images.counselheal.com/data/images/full/4421/tau-protein-alzheimers.jpg

[8] HENEKA M. T., CARSON M. J, KHOURY J. E., *et al*.: Neuroinflammation in Alzheimer's disease. *The Lancet Neurology*, 2015, 14 (4), p388-405. ISSN 1474-4422. DOI 10.1016/S1474-4422(15)70016-5.

[9] PAN M.H., LAI C.S. and HO C.T.: Anti-inflammatory activity of natural dietary flavonoids. *Food & function*, 2010, 1 (1), p15-31. ISSN 1756-4646. DOI 10.1039/c0fo00103a.

[10] DASURI K., ZHANG L. and KELLER J. N.: Oxidative stress, neurodegeneration, and the balance of protein degradation and protein synthesis. *Free radical biology & medicine*, 2013, 62, p170-185. ISSN 0891-5849. DOI 10.1016/j.freeradbiomed.2012.09.016.

[11] PATTEN D.A., GERMAIN M., KELLY M.A. and SLACK R.S.: Reactive oxygen species: stuck in the middle of neurodegeneration. *Journal of Alzheimer's disease*, 2010, 20 (2), p357-367. ISSN 1875-8908. DOI 10.3233/JAD-2010-100498.

[12] KIM G. H., KIM J. E., RHIE S. J. and YOON S.: The Role of Oxidative Stress in Neurodegenerative Diseases. *Experimental neurobiology*, 2015, 24 (4), p325-340. ISSN 2093-8144. DOI 10.5607/en.2015.24.4.325.

[13] SHALINI M.: Production of Reactive Oxygen Species and Its Implication in Human Diseases. *Springer India*, 2015, p3-15. ISBN 978-81-322-2035-0. DOI 10.1007/978-81-322-2035-0_1.

[14] SONG P. and ZOU M.H.: Regulation of NAD(P)H oxidases by AMPK in cardiovascular systems. *Free radical biology & medicine*, 2012, 52 (9), p1607-1619. ISSN 0891-5849. DOI 10.1016/j.freeradbiomed.2012.01.025.

[15] BABIOR B.M.: NADPH oxidase. *Current opinion in immunology*, 2004, 16 (1), p42-47. ISSN 0952-7915. DOI 10.1016/j.coi.2003.12.001.

[16] HYBERTSON B.M., GAO B., BOSE S.K. and MCCORD J.M.: Oxidative stress in health and disease: the therapeutic potential of Nrf2 activation. *Molecular aspects of*

medicine, 2011, 32 (4-6), p234-246. ISSN 0098-2997. DOI 10.1016/j.mam.2011.10.006.

[17] CALKINS M.J., JOHNSON D.A., TOWNSEND J.A., VARGAS M.R., DOWELL J.A., WILLIAMSON T.P., KRAFT A.D., LEE J.M., LI J. and JOHNSON J.A.: The Nrf2/ARE pathway as a potential therapeutic target in neurodegenerative disease. *Antioxidants & redox signalling*, 2009, 11 (3), p497-508. ISSN 1557-7716. DOI 10.1089/ARS.2008.2242.

[18] WANG X. and MICHAELIS E.K.: Selective neuronal vulnerability to oxidative stress in the brain. *Frontiers in aging neuroscience*, 2010, 2 (12), p1-13. ISSN 1663-4365. DOI 10.3389/fnagi.2010.00012.

[19] FERREIRA M.E., DE VASCONCELOS A.S., DA COSTA VILHENA T., DA SILVA T.L., DA SILVA BARBOSA A., GOMES A.R., DOLABELA M.F. and PERCÁRIO S.: Oxidative Stress in Alzheimer's Disease: Should We Keep Trying Antioxidant Therapies? *Cellular and molecular neurobiology*, 2015, 35 (5), p595-614. ISSN 1573-6830. DOI 10.1007/s10571-015-0157-y.

[20] WANG X., WANG W., LI L., PERRY G., LEE H.G. and ZHU X.: Oxidative stress and mitochondrial dysfunction in Alzheimer's disease. *Biochimica et biophysica acta*, 2014, 1842 (8), p1240-1247. ISSN 0006-3002. DOI 10.1016/j.bbadis.2013.10.015.

[21] JAMEEL E., UMAR T., KUMAR J. and HODA N.: Coumarin: A Privileged Scaffold for the Design and Development of Antineurodegenerative Agents. *Chemical biology & drug design*, 2016, 87, p21-38. ISSN 1747-0285. DOI 10.1111/cbdd.12629.

[22] VENUGOPALA K. N., RASHMI V. and ODHAV B.: Review on natural coumarin lead compounds for their pharmacological activity. *Biomed research international*, 2013, 2013, p1-14. ISSN 2314-6141. DOI 10.1155/2013/963248.

[23] BRÜHLMANN C., OOMS F., CARRUPT P.-A., TESTA B., CATTO M., LEONETTI F., ALTOMARE C. and CAROTTI A.: Coumarins Derivatives as Dual

Inhibitors of Acetylcholinesterase and Monoamine Oxidase. *Journal of Medicinal Chemistry*, 2001, 44 (19), p3195-3198. ISSN 0022-2623. DOI 10.1021/jm010894d.

[24] ROLLINGER J. M., HORNICK A., LANGER T., STUPPNER H. and PRAST H.: Acetylcholinesterase Inhibitory Activity of Scopolin and Scopoletin Discovered by Virtual Screening of Natural Products. *Journal of Medicinal Chemistry*, 2004, 47 (25), p6248-6254. ISSN 0022-2623. DOI 10.1021/jm049655r.

[25] KRISHNANAND M., HIMANSHU O. and NABO K.CH.: Estimation of antiradical properties of antioxidants using DPPH assay: A critical review and results. *Food Chemistry*, 2012, 130, p1036-1043. ISSN 0308-8146. DOI 10.1016/j.foodchem.2011.07.127.

[26] RAMPA A., TAROZZI A., MANCINI F., PRUCCOLI L., DI MARTINO R.M., GOBBI S., BISI A., DE SIMONE A., PALOMBA F., ZACCHERONI N. and BELLUTI F.: Naturally Inspired Molecules as Multifunctional Agents for Alzheimer's Disease Treatment. *Molecules*, 2016, 21 (5), p643. ISSN 1420-3049. DOI 10.3390/molecules21050643.

[27] TAROZZI A., MORRONI F., MERLICCO A., HRELIA S., ANGELONI C., CANTELLI-FORTI G. and HRELIA P.: Sulforaphane as an inducer of glutathione prevents oxidative stress-induced cell death in a dopaminergic-like neuroblastoma cell line. *Journal of neurochemistry*, 2009, 111 (5), p1161-1171. ISSN 1471-4159. DOI 10.1111/j.1471-4159.2009.06394.x.

[28] TAROZZI A., MORRONI F., BOLONDI C., SITA G., HRELIA P., DJEMIL A. and CANTELLI-FORTI G.: Neuroprotective effects of erucin against 6-hydroxydopamine-induced oxidative damage in a dopaminergic-like neuroblastoma cell line. *Internation Journal of Molecular Sciences*, 2012, 13 (9), p10899-10910. ISSN 1422-0067. DOI 10.3390/ijms130910899.

[29] ROBERSON M. and HARELL L.E.: Cholinergic activity and amyloid precursor protein metabolism. *Brain Res Rev* 1997, 25 (1), p50-69. ISSN 0006-8993. DOI: 10.1016/S0165-0173(97)00016-7.

[30] SCARPINI E., SCHELTERNS P., FELDMAN H.: Treatment of Alzheimer's disease; current status and new perspectives. *The Lancer Neurology*, 2003, 2 (9), p539-547. ISSN 1474-4422. DOI: 10.1016/S1474-4422(03)00502-7.

[31] TAROZZI A., BARTOLINI M., PIAZZI L. *et al* : From the dual function lead AP2238 to AP2469, a multi-target-directed ligand for the treatment of Alzheimer's disease. *Pharmacol Res Perspect*, 2014, 2 (2), e00023. ISSN 2052-1707. DOI: 10.1002/prp2.23. ISBN 10.1002/prp2.23.

[32] ALI M. D. Y., JANNAT S., JUNG H. A., CHOI R. J., ROY A. and CHOI J. S.: Anti-Alzheimer's disease potential of coumarins from Angelica decursiva and Artemisia capillaris and structure-activity analysis. *Asian Pacific Journal of Tropical Medicine*, 2016, 9 (2), p103-111. ISSN 2222-1808. DOI: 10.1016/j.apjtm.2016.01.014.

[33] KIM S.H., KANG K., ZHANG R., *et al*.: Protective effect of esculetin against oxidative stress-induced cell damage via scavenging reactive oxygen species. *Acta Pharmacol Sin*, 2008, 29 (11), p1319-1326. ISSN 1745-7254. DOI: 10.1111/j.1745-7254.2008.00878.x.

[34] THUONG P. T., HUNG T. M., NGOC T. M. et al.: Antioxidant activities of coumarins from Korean medicinal plants and their structure's activity relationships. *Phytotherapy research*, 2010, 24 (1), p101-106. ISSN 1099-1573. DOI: 10.1002/ptr.2890.

[35] WANG Ch., PEI A., CHEN J., YU H., SUN M., LIU Ch.and XU X.: A natural coumarin derivative esculetin offers neuroprotection on cerebral ischemia/reperfusion injury in mice. *Journal of Neurochemistry*, 2012, 121, p1007-1013. ISSN 1471-4159. DOI: 10.1111/j.1471-4159.2012.07744.x.